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Staphylococcus aureus cell envelope antigen is a new candidate for the induction of IgA nephropathy

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***Staphylococcus aureus* cell envelope antigen is a new candidate for the induction of IgA nephropathy.**

Background. IgA nephropathy is the most common form of glomerulonephritis worldwide. We previously reported a novel form of glomerulonephritis with glomerular IgA deposits following methicillin-resistant *Staphylococcus aureus* (*S. aureus*) infection. We investigated the role of *S. aureus* related antigens in the immunopathogenesis of IgA nephropathy by producing several monoclonal antibodies against *S. aureus* surface antigens and determining the epitopes of deposited antigens in patients with IgA nephropathy.

Methods. Cell membrane proteins were isolated from cultured *S. aureus.* Mouse monoclonal antibodies against these proteins were generated, and their target epitopes were determined by antibody affinity chromatography and amino acid sequence analysis, and by monoclonal antibody screening of *Escherichia coli* clones transfected with plasmids from the Lambda *S. aureus* Genomic Library. Renal biopsy specimens from 116 patients with IgA nephropathy and 122 patients with other forms of renal disease were examined for glomerular antigen depositions by immunofluorescence microscopy.

Results. The major antigen recognized by monoclonal antibodies against *S. aureus* cell membrane was identified as the *S. aureus* cell envelope antigen designated 'probable adhesin' (ACCESSION AP003131-77, Protein ID; BAB41819.1). In 68.1% (79/116) of renal biopsy specimens from patients with IgA nephropathy, *S. aureus* cell envelope antigen was localized in the glomeruli, and the data confirmed that *S. aureus* cell envelope antigen was co-localized with IgA antibody in the glomeruli. No deposition of this antigen was detected in the glomeruli of patients with non-immune complex deposit forms of glomerulonephritis.

Conclusion. S. aureus cell envelope antigen is a new candidate for the induction of IgA nephropathy.

Staphylococcus aureus (S. aureus) is both a normal and a pathogenic constituent of the oral floras that commonly colonize the surface of mucosal tissue and is kept in check by the mucosal and systemic immune systems [1]. Normal human sera routinely contain high concentrations of IgA and IgG antibodies against *S. aureus* [2]. However, *S. aureus* can cause postoperative wound infection, food poisoning, catheter-associated infection, septicemia, endocarditis, and sometimes toxic shock syndrome [3]. *S. aureus* is a unique bacterium that produces enterotoxins that act as superantigens [4, 5]. Additionally, on its cell surface, *S. aureus* displays protein A, which activates the proliferation of B cells [6].

Most reports associating staphylococcal infection with glomerulonephritis have emphasized two clinical forms: *Staphylococcus epidermidis (S. epidermidis)* bacteremia with infected ventriculo-jugular shunts, and *S. aureus* bacteremia with endocarditis [7]. These types of glomerulonephritis are caused by the deposition of immune complexes composed of IgG antibody and bacterial antigens in the glomeruli. We previously described a novel form of glomerulonephritis with nephrotic syndrome and rapidly progressive nephritic syndrome occurring after methicillin-resistant *Staphylococcus aureus* (MRSA) infection [8, 9]. This post-infection glomerulonephritis was characterized by mesangial proliferation with various degrees of crescent formation, and glomerular deposition of IgA, IgG, and C3. All patients with this form of glomerulonephritis showed both rapid increases in serum IgA and IgG levels and in specific T-cell receptor $V\beta^+$ subsets during the course of the disease. We found small but significant increases in the same T-cell receptor $V\beta^+$ subsets in some patients with IgA nephropathy (IgAN) [10]. Although the degree of post-MRSA infection glomerulonephritis was very marked, we hypothesize that this type of glomerulonephritis is in fact a severe form of IgA-related glomerulonephritis. IgAN is the most common form of glomerulonephritis and is one of the major causes of end-stage renal disease worldwide [11]. The renal survival rate at 20 years after the apparent onset of IgAN is estimated at 60–70% [12]. IgAN is thought to be induced by deposition of immune complexes containing IgA1 and

Key words: IgA nephropathy, *Staphylococcus aureus* cell envelope antigen, normal flora, adhesin, mucosal immunity, systemic immunity.

unidentified antigens in glomeruli [13]. Nearly fifty percent of patients with IgAN show an elevated serum IgA level, and many potential candidate antigens that might cause IgAN, including viruses, bacteria, and food antigens, have been reported [13–16]. In the present study, we investigated the possible role of *S. aureus* cell membrane antigens in the pathogenesis of IgAN by producing a series of monoclonal antibodies specific for *S. aureus* cell membrane epitopes. We characterized the antigens recognized by these antibodies and examined renal biopsy material from patients with IgAN to ascertain whether these antigens were localized on glomeruli.

METHODS

Patients

All patients were diagnosed by renal biopsy, clinical data and laboratory tests. IgAN was diagnosed by the presence of predominant deposition of IgA in the mesangium as observed by immunofluorescence microscopy and the proliferation of mesangial cells and matrix as observed by light microscopy.

A monoclonal antibody against *S. aureus* cell membrane antigen, whose production is described below, was used to stain renal biopsy specimens from 116 patients with IgAN and 122 patients with other forms of renal disease who served as controls. Among the control patients, 76 patients were diagnosed with non-immune complex types of renal disease: pauci-immune crescentic glomerulonephritis ($N = 8$), non-immune complex type of mesangial proliferative glomerulonephritis ($N = 8$), minimal change nephrotic syndrome ($N = 19$), focal segmental glomerulosclerosis ($N = 8$), minor lesions ($N = 19$), tubulointerstitial nephritis ($N = 8$), and benign nephrosclerosis ($N = 6$). Patients with acute glomerulonephritis ($N = 4$), membranous nephropathy ($N = 16$), membranoproliferative glomerulonephritis ($N = 2$), and lupus nephritis ($N = 10$) were classified as having immune complex types of renal disease. Patients with post-MRSA glomerulonephritis ($N = 4$) and Henoch-Schönlein purpura nephritis ($N = 10$) were classified as having IgA-related glomerulonephritis.

Preparation of *S. aureus* membrane antigens, enzyme-linked immunosorbent assay (ELISA) antigen and other bacterial lysates

S. aureus strain 8325 was cultured to the stationary phase in Brain Heart Infusion medium (BHI; Difco, Becton Dickinson Co., Ltd., Cockeysville, MD), shaken at 37°C overnight, and centrifuged at 12,000 × *g* at 4°C for 30 min, followed by washing with sterilized TE buffer (10 mM Tris-HCl+1 mM EDTA, pH 8.0). The pellet was then suspended in 40 mL of TE buffer, and 80 μL of lysostaphin (2 mg/mL dissolved in 20 mM NaOAC; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 4°C was added. The mixtures was incubated at 37°C for 45 min for bacteriolysis and then was centrifuged at 28,000 × *g* at 4°C for 90 min. The precipitate was centrifuged at 100,000 × *g* at 4°C for 1 hr to pellet the cell membrane fraction and used as crude *S. aureus* membrane antigens. Then, *S. aureus* crude membrane antigens were prepared for ELISA antigen by the absorption of protein A with human IgG-coated Sepharose 4B.

The other bacterial crude lysates, including *Staphylococcus epidermidis, Enterococcus faecalis, Enterococcus gallinarum, Enterococcus casseleflarus, Enterococcus raffinosus, Streptococcus oralis, Streptococcus pneumoniae,* Group A *Streptococcus,* Group G *Streptococcus, Neisseria subflava, Escherichia coli (E. coli), Citrobacter freundii, Klebsiella oxytoca, Klebsiella pneumoniae, Enterobacter cloacae, Serratia mariesiens, Proteus mirabillis, Pseudomonas aeruginosa*, were prepared as *S. aureus* strain 8325, which did not be purified for the cell membrane fraction.

Preparation of mouse monoclonal antibodies against *S. aureus* membrane antigens

Four-week-old BALB/c mice were immunized with 200 μg of *S. aureus* crude membrane antigens in Freund's Incomplete Adjuvant twice weekly for 3 months. After sacrifice by heart puncture under anesthesia, spleen cells were obtained from the immunized mice and fused with NS1 cells by the polyethylene glycol method [17]. After cloning hybridomas by limiting dilution, monoclonal antibody-producing clones were obtained.

Purification of *S. aureus* membrane antigen epitope

A HiTrap NHS-activated column (Amersham Pharmacia Biotech AB) was conjugated with protein G-purified mouse ascites containing described above prepared monoclonal antibody. After washing and deactivation of 3 cycles of washing with 0.5M ethanolamine, pH 8.3, and 0.1 M acetate, 0.5 M NaCl, pH 4.0, was then washed with 20 mM PBS, pH 7.0, to adjust the pH to 7.0. The membrane antigens of *S. aureus,* which were prepared by the absorption of protein A with human IgG-coated Sepharose 4B, were applied to the column. After washing with PBS, pH 8.0, elution of the antigen was performed with 0.15 M glycine-HCl, pH 2.0, and the pH of the eluate was neutralized with 0.15 M glycin-NaOH buffer, pH 10.0.

Western blot analysis

Crude *S. aureus* membrane antigens, the protein A free antigen (ELISA antigen), protein A (Cappel, ICN Biomedicals, Inc. Aurora, Ohio), and bacterial lysates were size-fractionated on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) plates, transferred to nitrocellulose membranes, and reacted with primary and secondary antibodies. After

washing, the nitrocellulose membranes were incubated with ECL+PLUS (RPN 2132; Amersham Pharmacia Biotech AB, Uppsala, Sweden) at room temperature for 60 min before photographic exposure. For characterization of ELISA antigen, serum from the IgAN patient was used as the primary antibody and horseradish peroxidase conjugated (HRP) –antihuman IgG, IgA (Dako, Glostrup, Denmark), or chicken HRP-anti-protein A (IMMUNOSYSTEM AB, Uppsala, Sweden) were used as the secondary antibodies. For analysis of human antibodies against *S. aureus* membrane antigens, the sera of patients with IgAN patients and normal control were used as the primary antibodies, and HRP-anti-human IgA was used as the secondary antibodies. For characterization of mouse monoclonal antibodies against *S. aureus* membrane antigens, described above prepared mouse monoclonal antibodies against *S. aureus* membrane antigens were used as the primary antibodies, and chicken HRP-anti-mouse IgG (chicken IgY) (OME Concepts Inc., Toms River, NJ, USA) was used as the secondary antibodies. For analysis of the monoclonal antibody with other bacteria cross-reactivity, mouse monoclonal antibody against *S. aureus* membrane antigens and mouse IgG isotype control (Medical & Biological Laboratories Co. Ltd, Nagoya, Japan) were used as the primary antibody and chicken HRP-anti-mouse IgG was used as the secondary antibody.

Quantitative analysis of anti-*S. aureus* antibodies by ELISA

IgG and IgA class antibody titers against *S. aureus* in sera from various types of renal diseases and normal individuals were measured by ELISA. ELISA plates were coated with 50 μL (5 μg/mL) of the protein A free antigen were coated in 20 mM carbonate buffer, pH 9.8, and incubated overnight at 4°C and washed 5 times with washing buffer (0.01M PBS, pH 8.0, plus 0.05% Tween-20 (PBS-Tween-20). Next, 250 μL of blocking buffer (0.5% bovine serum albumin (BSA) in PBS-Tween-20) were added to each well, and the resulting mixture was incubated at room temperature for 1 hour. Plates were then washed with washing buffer. Fifty microliters of a 1/100 dilution of serum samples were added to the wells and incubated at 37°C for 1 hour, then washed 5 times with washing buffer. Fifty microliters of a 1/1000 dilution of rabbit HRP-anti-human IgA and IgG (Dako) was added to the wells and incubated for 1 hour at 37°C, then washed 5 times with washing buffer. Fifty microliters of substrate ABTS (2, 2-azino-di (3-ethylbenthiazoline) sulfonic acid Zymed Laboratories, Inc., CA, USA) was added to the wells and incubated at room temperature for 30 minutes, and 50 μL of stopping buffer (2 mmol/L NaN_3) was added, and then measured by an immunoreader with 405nM/540nM filtering. The antibody titers were expressed as OD units.

Amino acid sequence analysis

Amino acid sequence analysis was performed by automated Edman degradation of peptides generated by chemical or enzymatic cleavage of the reduced and S-carboxymethylated protein. A homology search was carried out using the BLAST database [18].

Identification of monoclonal antibody epitopes

The Lambda Zap II vector containing an *S. aureus* library (RN450 strain; Stratagene, La Jolla, CA, USA) was used to transform *E. coli* cells (XL1-Blue MRF' host strain). After induction with isopropyl-1-thio-β-D-galactopyranoside (IPTG), *E. coli* clones that reacted with the monoclonal antibody described above were screened for *S. aureus* membrane antigens. The plasmid DNA from clone was purified using a purification kit (Gene Clean II kit; Bio 101, La Jolla, CA, USA) and amplified by polymerase chain reaction (PCR) with M13 primers (5′-GGAAACAGCTATGACCATG-3′ and 5′-GTAAAACGACGGCCAGT-3′) using a dGTP sequence kit (dGTP Big Dye Terminator; ABI Prism, PE, Applied Biosystems, Foster City, CA, USA). The PCR conditions were 25 cycles of denaturation at 90°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes. The DNA sequence of the PCR product was analyzed with an automated DNA sequencer (ABI 310 Genetic Analyzer; Applied Biosystems).

Antigen detection by immunofluorescent examination of renal biopsy specimens

Renal biopsy specimens were examined by routine light, electron, and immunofluorescence microscopy. A portion of each specimen was frozen with n-hexane in solid carbon dioxide-acetone for immunofluorescence microscopy. Frozen 4 μm tissue sections were stained in the standard manner using mouse monoclonal antibody to *S. aureus* membrane antigen and fluorescein isothiocyanate-rabbit anti-mouse IgG antibody (Sigma). To study whether *S. aureus* membrane antigen and human IgA antibody were co-localized in renal biopsy specimens, tissue sections were stained with a mixture of mouse monoclonal antibody to *S. aureus* membrane antigen and rabbit anti-human IgA (Sigma) as primary antibodies, and with a mixture of Alexa 488-labeled goat anti-mouse IgG antibody (Molecular Probes, Inc., Eugene, OR, USA) and Alexa 594-labeled goat anti-rabbit IgG antibody (Molecular Probes, Inc.) as secondary antibodies. The blocking buffer was 2% normal goat serum in 0.05% Tween 20-PBS. As a negative control, a mouse IgG1 isotype control (Medical & Biological Laboratories Co. Ltd.) was used. An immunofluorescence microscope (Olympus BX51, Olympus Optical Co. Ltd., Tokyo, Japan), ORCA-1394 digital camera (Hamamatsu Photonics Co. Ltd., Hamamatsu,

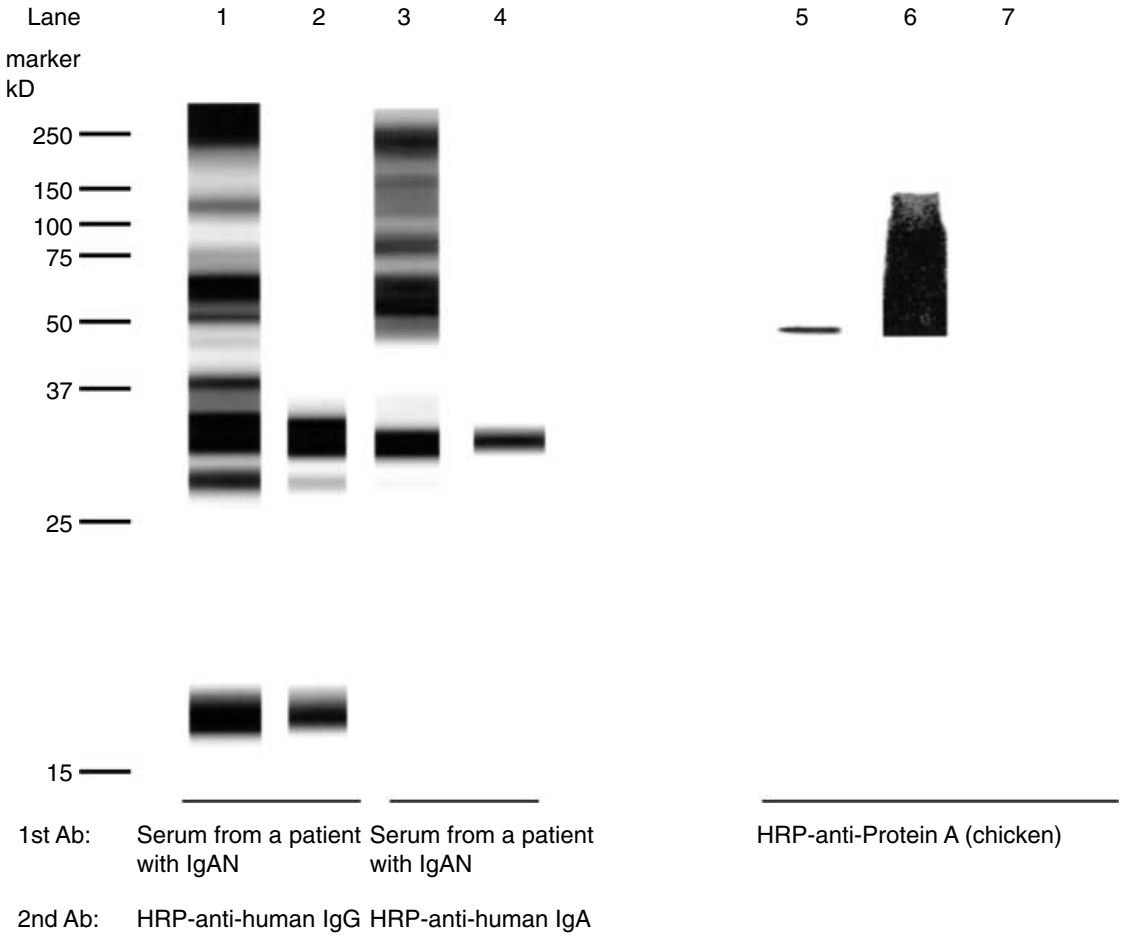


Fig. 1. Western blot analysis of *S. aureus* antigens. Lanes 1 and 3: crude membrane antigen. Lanes 2 and 4: antigen prepared by absorption of protein A with human IgG-coated Sepharose 4B. Staining was with a IgA nephropathy patient's serum (1st Ab), followed by staining with HRP-anti-human IgG (lanes 1 and 2) or HRP-anti-human IgA (lanes 3 and 4) (2nd Ab). Lane 5: Protein A. Lane 6: crude membrane antigen. Lane 7: antigen prepared by absorption of protein A with human IgG-coated Sepharose 4B. Lanes 5, 6 and, 7 were stained with chicken anti-Protein A.

Japan), and AQUA-Lite software (Hamamatsu Photonics Co. Ltd.) were used to study antigen and antibody co-localization.

Statistical analysis

All data were statistically analyzed by the analysis of variance (ANOVA), the Mann-Whitney test, and the chi-square test. A P value of less than 0.05 was considered to be significant.

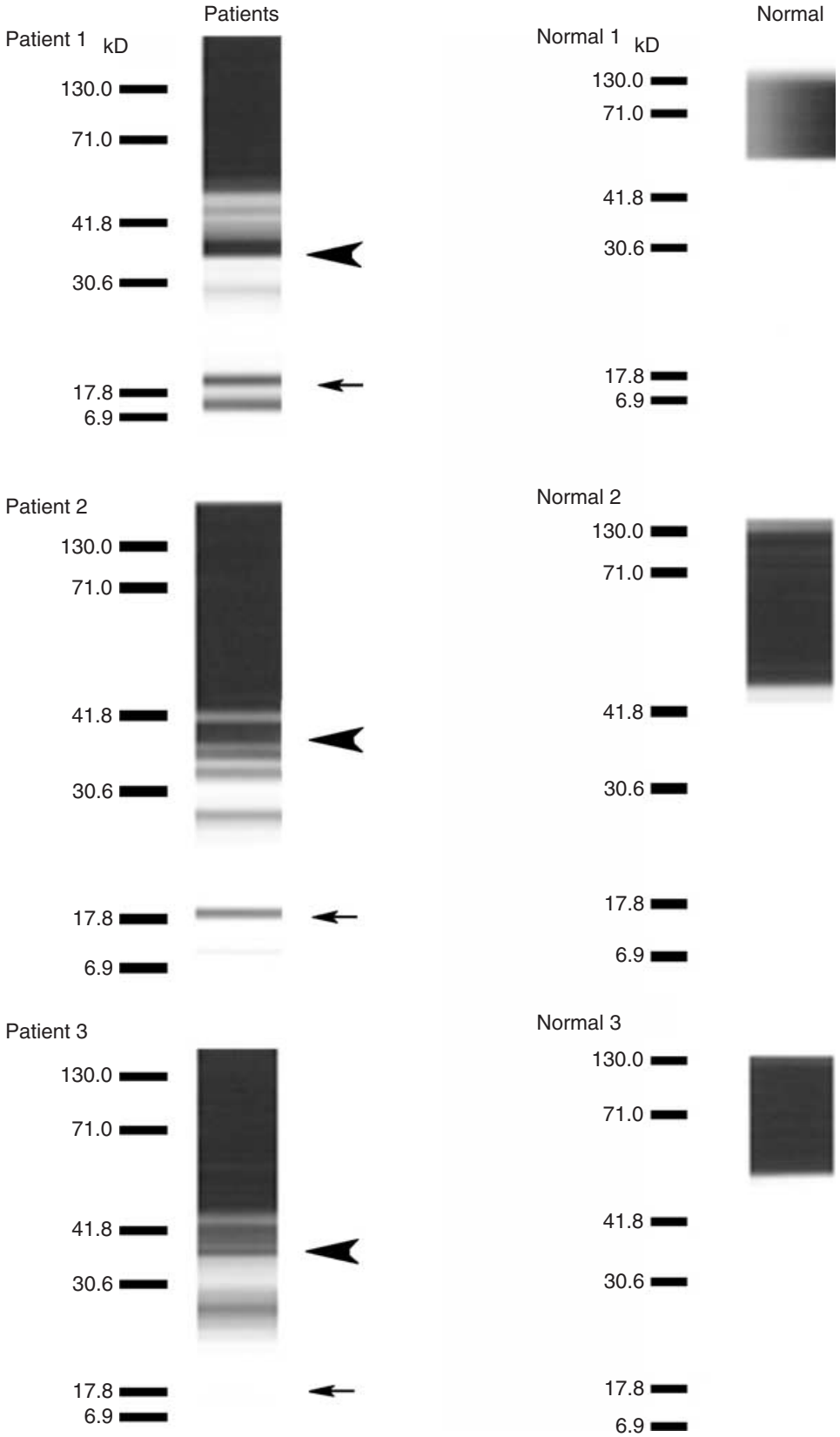
RESULTS

Characterization of crude *S. aureus* membrane antigens and the antigen for ELISA

Crude *S. aureus* membrane antigens were prepared from *S. aureus* 8325. Then, *S. aureus* crude membrane antigens were purified for ELISA antigen by the absorption of protein A with human IgG-coated Sepharose 4B. To characterize crude *S. aureus* membrane antigens and ELISA antigen, immunoblot analyses were performed (Fig. 1). In lanes 1 and 3 (crude membrane antigen), numerous bands, ranging high to low molecular weight, were observed. However, in lanes 2 and 4 (the antigen for ELISA), we could not detect bands larger than 50 kD. In lane 5 (protein A of *S. aureus*), a narrow band was observed at 50 kD, and in lane 6 (crude membrane antigen), a broad band, ranging 50 to 150 kD was observed. In lane 7 (antigen used in ELISA), we failed to detect any band.

Analysis of human antibodies against crude *S. aureus* membrane antigens

Crude *S. aureus* membrane antigens were subjected to electrophoresis, and we applied the sera of patients with IgAN and control patients, followed by staining with HRP-labeled anti-human IgA antibody (Fig. 2). Upon application of sera from patients with IgAN, large (>50 kD) and relatively small band especially 35 kD and 17 kD were frequently revealed. In contrast, when the sera from control patients were applied, only large bands (>50 kD) were detected.



1st antibodies: sera from patients and control subjects

2nd antibody: HRP-anti-human IgA

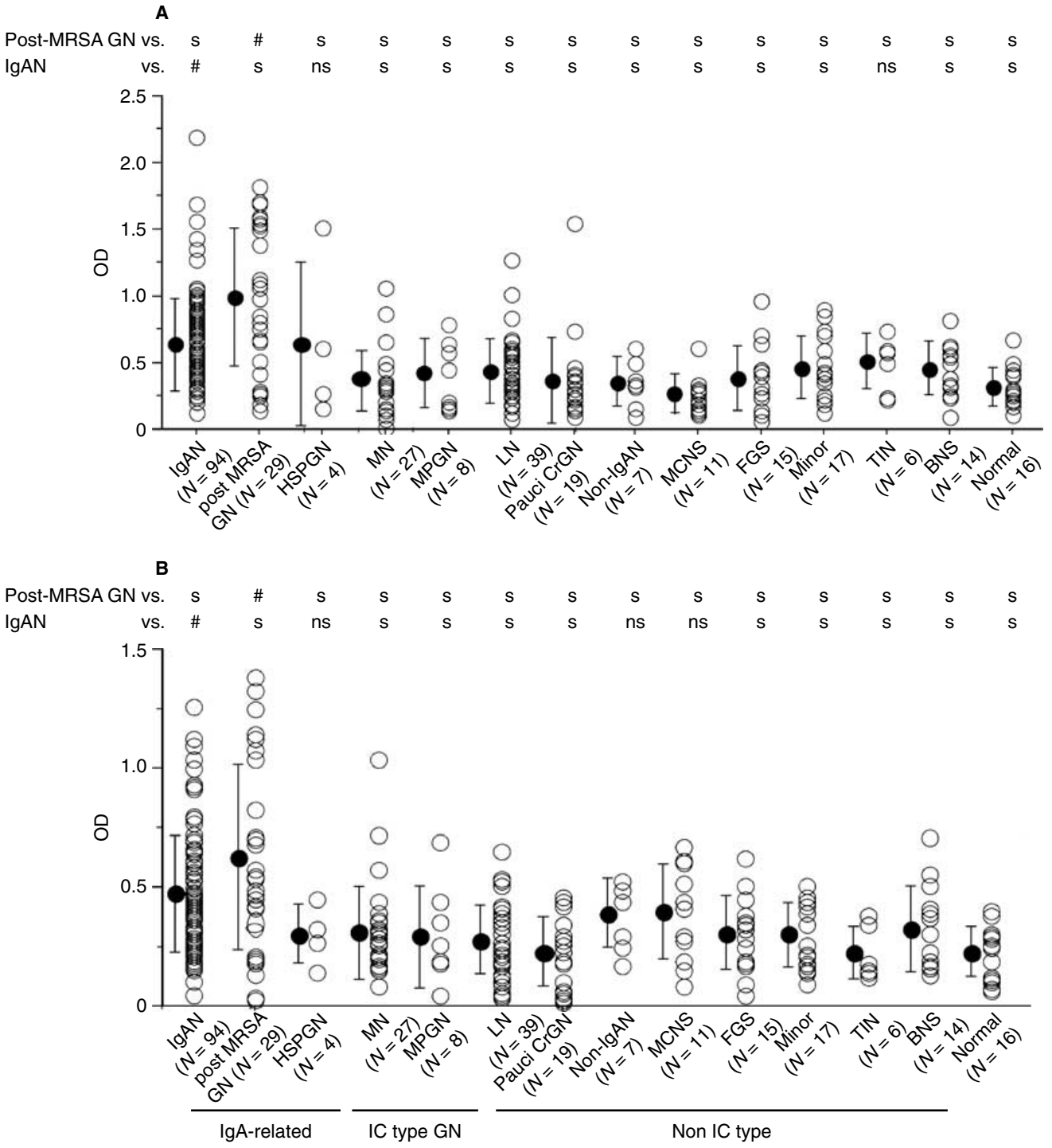


Fig. 3. Antibody titers to *S. aureus* in IgAN and other types of renal diseases by ELISA. The upper schema indicates anti-*S. aureus* antibodies (IgG class). The lower schema indicates anti-*S. aureus* antibodies (IgA class). S: significant difference ($P < 0.05$). ns: not significant difference. HSPGN: Henoch-Schönlein purpura nephritis, MN: Membranous nephropathy, MPGN: menbranoproliferative GN, LN: lupus nephritis, Pauci Cr GN: pauci-immune crescentic GN, non-IgAN: non-immune deposit mesangial proliferative GN, MCNS: minimal change nephritic syndrome, FGS: focal glomerular sclerosis, minor: minor change, TIN: tubulointerstitial nephritis, BNS: benign nephrosclerosis. Solid circles indicate mean ± SD.

←

Fig. 2. Western blot analysis in sera from patients and controls for antibodies to *S. aureus* membrane antigen. Crude *S. aureus* membrane antigen was analyzed in each lane and probed with sera from three control subjects and three patients with IgAN, followed by staining with HRP-anti-human IgA. In the patients with IgAN, both large molecular weight (>50 kD) and relatively smaller molecular weight bands, in particular at 35 kD (arrow head) and 17 kD (arrow), were frequently detected. In contrast, in the three control subjects, only bands larger than 50 kD were detected.

Quantitative analysis of anti-*S. aureus* antibodies (IgG and IgA)

In order to measure the titer of anti *S. aureus* antibodies in patients with renal diseases and normal individuals, ELISA analysis were performed. ELISA plates were coated with described above ELISA antigen. Ninety-four serum samples from patients with IgAN, 196 serum samples from patients with various types of renal diseases, and 16 serum samples from normal individuals were examined (Fig. 3). Both IgG and IgA titers to *S. aureus* in the sera from patients with IgAN and post-MRSA glomerulonephritis were significantly higher than those in other forms of renal disease and normal individuals.

Characterization of mouse monoclonal antibodies against *S. aureus* membrane antigen

We generated several monoclonal antibodies against the crude membrane antigen of *S. aureus* in BALB/c mice. Five clones, S9D6 (IgG2aκ), S1D7 (IgG1κ), S1B9 (IgG1κ), S1D6 (IgG1κ), and S10D4 (IgG2bκ), reacted with a 35 kD band of *S. aureus* membrane antigen, and 1 clone, S9C7 (IgG2aκ), reacted with a 17 kD band. The five monoclonal antibodies reacted with the same 35 kD *S. aureus* membrane antigen, based on DNA sequence analysis of the antigen recognized by each monoclonal antibody. One of the monoclonal antibodies that reacted with the 35 kD band (Fig. 4), S1D6, was used as a representative antibody in the following experiments.

Cross-reactivity of the monoclonal antibody, S1D6, with the other bacteria

To investigate the cross-reactivity of monoclonal antibody S1D6 with the other bacteria, immunoblot analyses were performed (Fig. 5). The monoclonal antibody against *S. aureus* membrane antigen, S1D6, cross-reacted with only the bacterial lysates of *S. epidermidis* (35 kD band), group G *Streptococcus* (150 kD band), and *Streptococcus pneumoniae* (150 kD band). However, in lane 18 in which we had applied group G *Streptococcus* cell lysate and stained it with mouse IgG1 isotype control, a 150 kD band was seen.

Determination of *S. aureus* membrane antigenic epitope

In order to determine *S. aureus* membrane antigenic epitope, antigen, prepared by the absorption of protein A with human IgG-coated Sepharose 4B, was purified by S1D6 antibody affinity chromatography. In the analysis of SDS gels stained with coomassie G 250 (Bio-Rad laboratories, Hercules, CA) staining, there was no 50 kD band, and a strong 35 kD band was observed in lane 1 (the antigen prepared by the absorption of protein A with human IgG-coated Sepharose 4B). In lane 2 (eluate from the HiTrap affinity column), a single 35 kD band

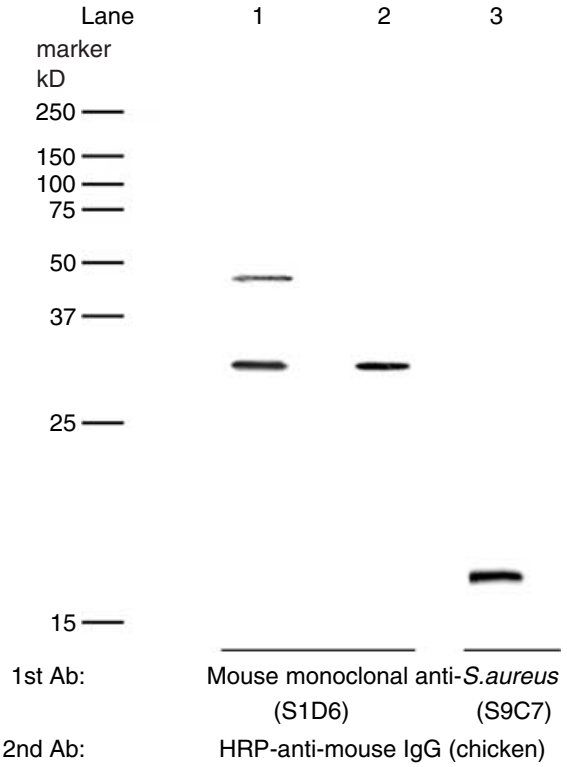
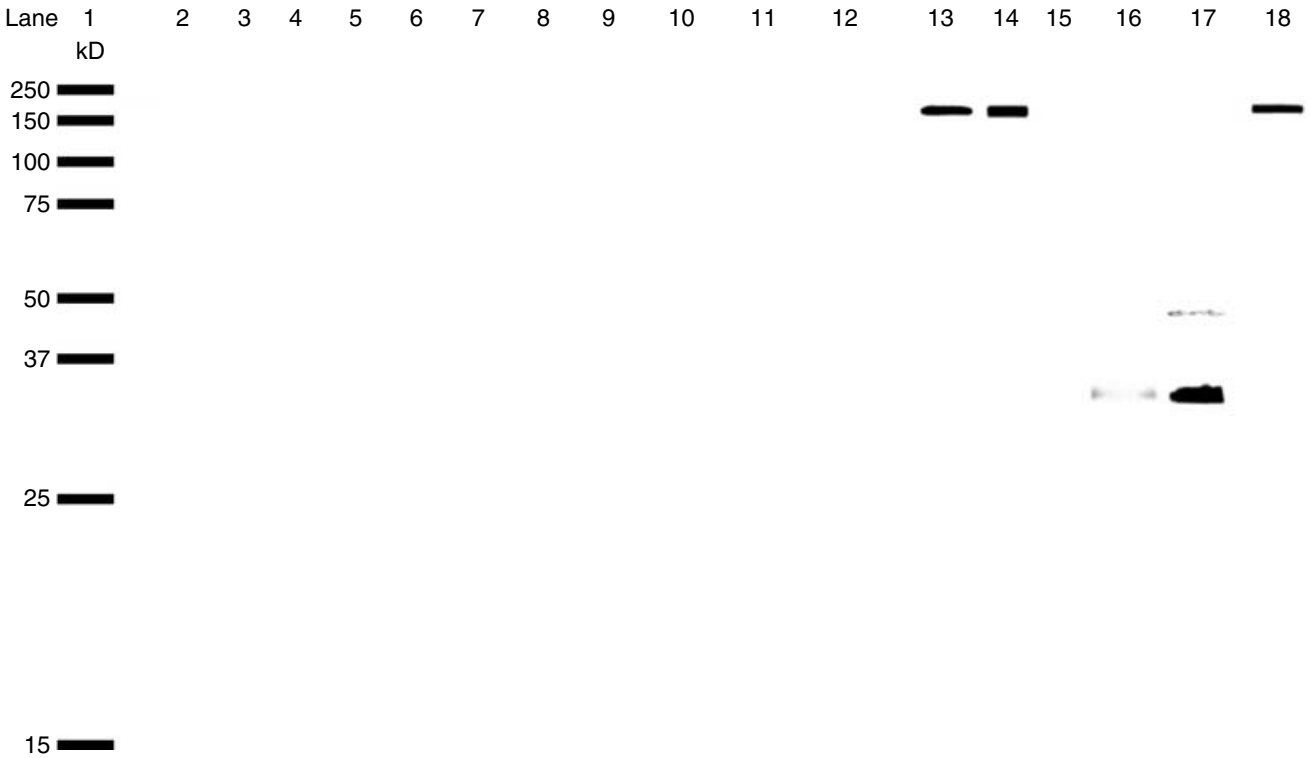


Fig. 4. Western blot analysis using 2 representative monoclonal antibodies against *S. aureus*. Lane 1: crude *S. aureus* cell membrane antigen. Lanes 2 and 3: antigen prepared by absorption of protein A with human IgG-coated Sepharose. Staining was with mouse monoclonal antibody S1D6 (IgG1-κ) or S9C7 (IgG2a-κ), (1st Ab), followed by staining with HRP-anti-mouse IgG (2nd Ab). A monoclonal antibody, S1D6, reacted with a 35 kD band of crude *S. aureus* cell membrane antigens, and a clone, S9C7, reacted with a 17 kD band.

was observed. A 50 kD band was seen in lane 3 (protein A) (Fig. 6). Amino acid sequence analysis of the eluate from the nitrocellulose membranes was performed, revealing a sequence of 20 amino acids (NVGGDNVDIHSIVPVGQDPH).

Characterization of *S. aureus* membrane antigenic epitope

For identification of epitopes, monoclonal antibody screening of *E. coli* clones transfected with plasmids from the Lambda *S. aureus* genomic library was performed. *E. coli* clones that reacted with monoclonal antibody S1D6 were screened for *S. aureus* membrane antigens. Immunoblot analysis showed that #26 clone reacted with the monoclonal antibody. The clone plasmid DNA was purified. The #26 DNA sequence was performed and a homology search for the sequence of this antigen was also done using the entire *S. aureus* genome database and the program BLAST. The sequence was identified as *S. aureus* cell envelope antigen, 'probable adhesin' (34.7 kD, Accession AP003131-77, Protein ID; BAB41819.1) [18, 19]. The 20 amino acids sequence



1. marker
2. *Citrobacter freundii*
3. *Enterobactor*
4. *Escherichia coli*
5. *Haemophilus influenzae*
6. *Haemophilus parainfluenzae*
7. *Klebsiella*
8. *Proteus*
9. *Pseudomonas*
10. *Serratia*
11. *Neisseria*
12. *Enterococcus faecalis*
13. *Streptococcus pneumoniae*
14. *Group G Streptococcus*
15. *Streptococcus oralis*
16. *Staphylococcus epidermidis*
17. *Staphylococcus aureus 8325*
18. *Group G Streptococcus*

Fig. 5. Cross-reactivity of monoclonal antibody S1D6 with the other bacteria. Lane 1: marker mixture. Lanes 2–17: bacterial lysates. Lane 18: Group G *Streptococcus* (which was also in lane 14). Staining was with mouse monoclonal antibody S1D6 (lanes 2–17) or mouse IgG isotype control (lane 18), followed by HRP-anti-mouse IgG (chicken).

(NVGGDNVDIHSIVPVGQDPH), determined as *S. aureus* membrane antigenic epitope, was included in the amino acid sequence of the 'probable adhesin' (Fig. 7). The sequence of this protein showed high homology to that of the *S. epidermidis* lipoprotein and *Streptococcus pneumoniae* PsaA.

Immunofluorescent examination of renal tissues

The monoclonal antibody S1D6 was applied to renal biopsy specimens from 116 patients with IgAN and 122 patients with other renal diseases (Table 1). The *S. aureus* cell envelope antigen was detected on the glomeruli of 68.1% (79/116) of the patients with IgAN (Fig. 8B), while it was not detected in the renal biopsy specimens from any of the patients with non-immune complex types of renal disease (0/76). However, this antigen was detected on the glomeruli of some patients with other immune complex types of glomerular disease. On the other hand, we failed to find any antigen in the glomeruli of patients with IgAN detected by a monoclonal antibody (S9C7) reacting with the 17 kD band. We also showed the double-staining of *S. aureus* membrane antigen and anti-human IgA antibody (Fig. 8C). Deposits of *S. aureus* membrane antigen and IgA antibody are both detected in the mesangial areas.

DISCUSSION

The major finding in this study was that deposition of an *S. aureus* cell envelope antigen was detected on the glomeruli of nearly 70% of the patients with IgAN, by staining with a monoclonal antibody that had been newly developed against a 35 kD antigen on the cell surface of *S. aureus*. We characterized the antigen by both monoclonal antibody affinity chromatography and amino acid sequence analysis, and we identified it as the *S. aureus* cell envelope antigen, 'probable adhesin' (Accession AP003131-77, Protein ID; BAB41819.1). We confirmed the identity of the antigen by monoclonal antibody screening of clones of *E. coli* transfected with plasmids

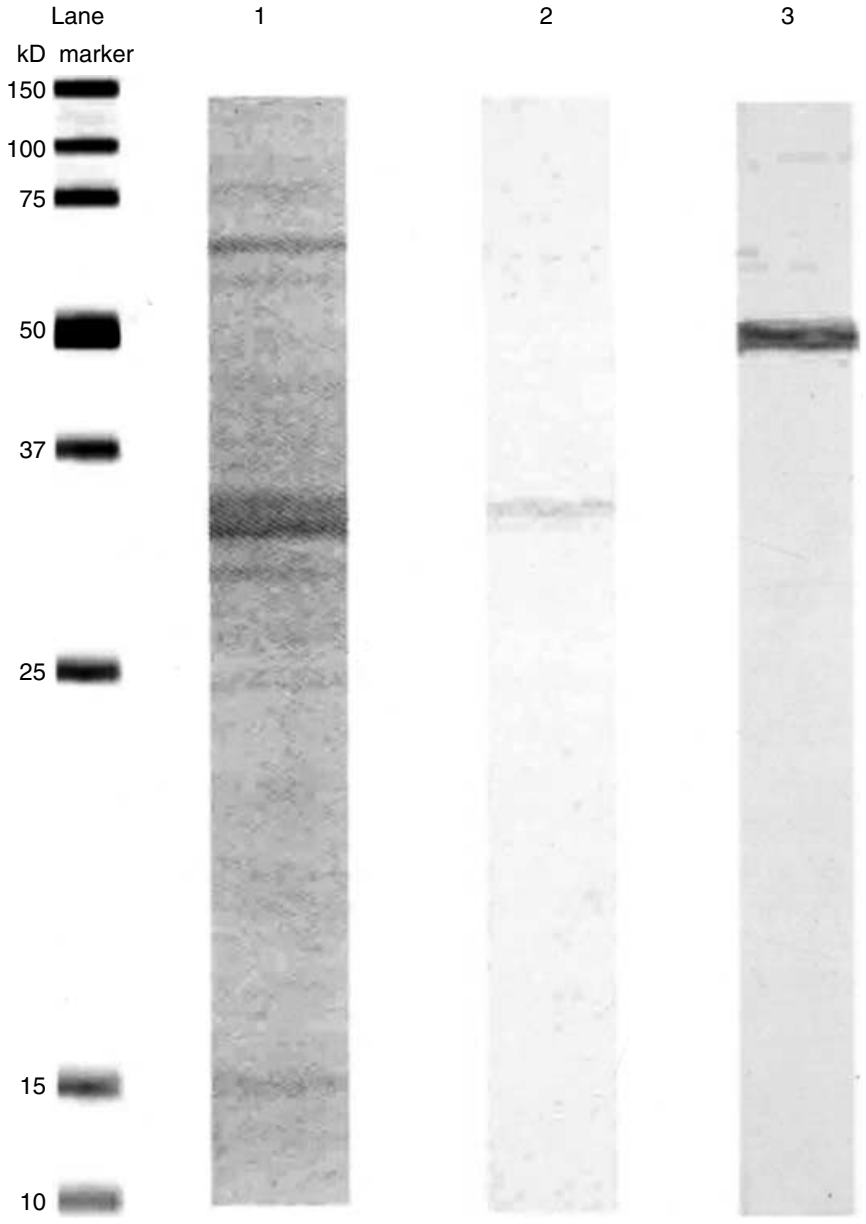


Fig. 6. Analysis of applied antigen to the Hi-Trap affinity column and the eluate from the HiTrap column by SDS-PAGE and coomassie staining. Lane 1: applied antigen, prepared by absorption of protein A with human IgG-coated Sepharose. Lane 2: Eluate from anti-*S. aureus* antibody-coated affinity column. Lane 3: protein A. SDS-gel was stained with Coomassie G 250.

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  1 MKKLVPLLLA LLLLVAACGT GGKQSSDKSN GKLKVVTTNS ILYDMAKNVG GDNVDIHSIV
 61 PVGQDPHEYE VKPKDIKKLT DADVILYNGL NLETGNGWFE KALEQAGKSL KDKKVIAVSK
121 DVKPIYLNGE EGNKDKQDPH AWLSLDNGIK YVKTIQQTFI DNDKKHKADY EKQGNKYIAQ
181 LEKLNNDSKD KFNDIPKEQR AMITSEGAFK YFSKQYGITP GYIWEINTEK QGTPEQMRQA
241 IEFVKKHKLK HLLVETSVDK KAMESLSEET KKDIFGEVYT DSIGKEGTKG DSYYKMMKSN
301 IETVHGSMK
(34.7-kD, ACCESSION AP003131-77, Protein ID; BAB41819.1).
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Fig. 7. Amino acid sequence of *S. aureus* cell membrane antigen. Amino acid sequence of *S. aureus* cell envelope antigen (34.7 kD, Accession AP003131-77, Protein ID; BAB41819.1) was shown. The bold amino sequence was as same as the 20 amino acids of the determined *S. aureus* membrane antigenic epitope form HiTrap affinity column in Fig. 6.

of a Lambda genomic library of *S. aureus* DNA clones [19].

Using Western blot analyses, we also demonstrated that 35 kD and 17 kD bands were strongly positive in the sera from patients with IgAN. For accurate comparison, we measured the titers of anti-*S. aureus* antibodies in various types of glomerulonephritis by ELISA. In the sera from patients with IgAN and post-MRSA

Table 1. Glomerular deposition of *S. aureus* antigen

Diagnosis[a]	No. of patients			
	Total	Antigen positive	Antigen negative	Percent positive %
IgA nephropathy	116	79	37	68.1
IgA nephropathy-related types				
Post-MRSA glomerulonephritis	4	3	1	75.0
Henoch-Schönlein purpura nephritis	10	6	4	60.0
Immune complex types				
Acute glomerulonephritis	4	0	4	0.0
Membranous nephropathy	16	1	15	6.3
Membranoproliferative glomerulonephritis	2	0	2	0.0
Lupus nephritis	10	3	7	30.0
Nonimmune complex types				
Pauci-immune crescentic glomerulonephritis	8	0	8	0.0
Non-IgA nephropathy	8	0	8	0.0
Minimal change nephrotic syndrome	19	0	19	0.0
Focal segmental glomerular sclerosis	8	0	8	0.0
Minor lesions	19	0	19	0.0
Tubulointerstitial nephritis	8	0	8	0.0
Benign nephrosclerosis	6	0	6	0.0

[a]Glomerular deposition of *S. aureus* membrane antigen was detected by immunofluorescent staining with monoclonal antibody, S1D6. Fluorescein isothiocyanate-rabbit antimouse IgG antibody was used as the secondary antibody.

glomerulonephritis, titers of anti-*S. aureus* antibodies (IgG and IgA classes) were significantly higher than those in other types of GN and in normal individuals. The target antigens were 35 and 17 kD antigens, and it is likely that we detected the antibodies to same antigens by Western blotting as by ELISA. These results suggest that both 35 kD and 17 kD antigens of *S. aureus* may have strong immunogenicity in many patients with IgAN. Therefore, immune complexes composed of *S. aureus* antigens, especially 35 and 17 kD antigens and their antibodies, may play a role in the pathogenesis of IgAN.

We have detected colonies of *S. aureus* on the oral and nasal mucosal surfaces of some patients with IgAN as well as on normal individuals (unpublished data). According to Kluytmans et al [3], there are three distinguishable patterns of *S. aureus* carriage: approximately 20% of individuals almost always carry one strain; a large proportion of the population (60%) harbors *S. aureus* intermittently; and the remaining 20% almost never carry *S. aureus*. The mean carriage rate in the general population is 37.2%; therefore, many people may potentially develop IgAN. Colonization and inversion of bacteria in the blood circulation are thought to be influenced by the balance between the mucosal and systemic immunities. However, the mechanism by which *S. aureus* initiates colonization of host tissues is not completely understood. *S. aureus* can adhere to components of the extracellular matrix in the host. Adherence is mediated by protein adhesins on the surface of *S. aureus*; in most cases, the protein adhesins

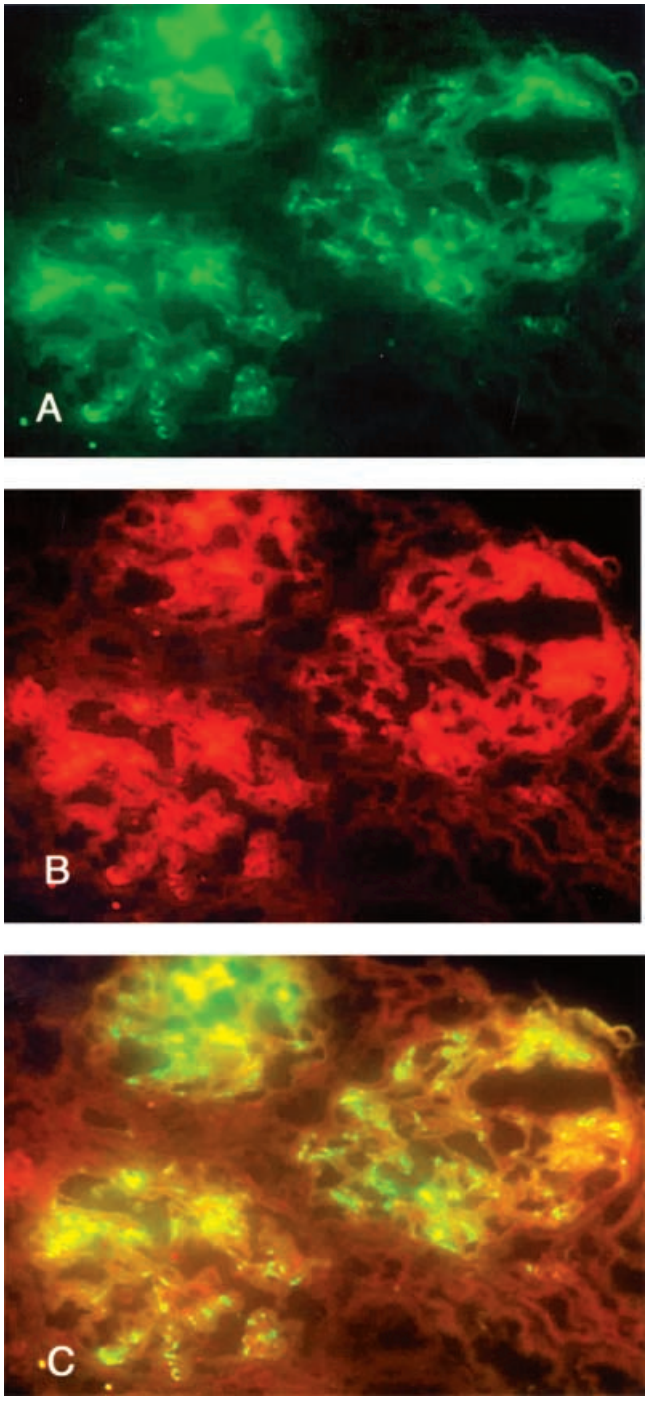

Fig. 8. Co-localization of *S. aureus* envelope antigen and IgA antibody in the glomeruli of a patient with IgA nephropathy. (*A*) Immunofluorescence staining with rabbit anti-human IgA antibody. Predominant mesangial depositions (green) are noted in the three glomeruli. (*B*) Immunofluorescence staining of *S. aureus* membrane antigen with monoclonal antibody (S1D6). Deposits (red) are mainly observed in the glomerular mesangial area. (*C*) Double-staining of *S. aureus* membrane antigen and antihuman IgA antibody. Deposits of *S. aureus* membrane antigen and IgA antibody (yellow) are observed in the mesangial areas. This indicates the co-localization of antigen and antibody.

are covalently anchored to the cell wall peptidoglycan and recognize the family of adhesive matrix molecules [20]. Our data indicate that our candidate antigen for the pathogenesis of IgAN is the newly reported 'probable adhesin' [19]. Fibronectin has been reported to be present in the circulating immune complexes of patients with IgAN [21]. However, not all patients with IgAN have IgA-fibronectin complexes [22], and differences in the composition of IgA immune complexes are still unclear. In this study, we have identified the antigen that was deposited on the glomeruli of many patients with IgAN as 'probable adhesin' [19]. We believe that this antigen and the antibodies specific for it constitute the complexes in the circulation and that these complexes are deposited on the glomeruli.

IgA, especially IgA1, has unique characteristics. Recently, there have been many reports on the role of the galactose-deficient hinge region of IgA1 in the pathogenesis of IgAN; in particular, characteristics of IgA1, such as the instability of its structure [23, 24], self-aggregation [25, 26], adhesion to extracellular matrix [25, 26], production of autoantibodies (to IgG or GalNAc residual) [27, 28], and its glomerulophilic characteristics [29] have been reported. Considering these reports, we speculate that *S. aureus* antigen-IgA1-immune complexes are easily deposited in the glomeruli, because IgA1 has these unique characteristics.

Regarding the immunogenetic background of IgAN, there have been reports of HLA linkage with this disease [13]. Recently, Gharavi et al [30] reported that familial IgAN is linked to 6q22-23. The immunologic status of patients with IgAN, especially regarding the Th1 and Th2 types, has been reported [31]. Patients with IgAN have Th2 predominance in their peripheral Tlymphocytes and in their cytokine profiles [32]. We speculate that these findings are related to normal or pathological bacterial colonization and abnormal immune response to these antigens.

It is possible that our monoclonal antibody cross-reacted with proteins on the cell envelope of other bacteria such as *S. epidermidis* lipoprotein and *Streptococcus pneumoniae* PsaA, both of which show high homology to this antigen. We analyzed the cross-reactivity of this monoclonal antibody to the bacterial lysates of several normal and pathogenic flora, including *S. epidermidis* and *Streptococcus pneumoniae*, by the Western blot method and found that there was no cross-reactivity of this antibody with the bacterial lysates of other bacteria except for a weak 35 kD band in *S. epidermidis* and a strong 150 kD band in group G *Streptococcus* and *Streptococcus pneumoniae*. However, when we stained the bacterial lysate from *Streptococci* with mouse IgG1 isotype control by Western blot analysis, a 150 kD band was also detected. Therefore, these 150 kD bands were thought to be nonspecific and may represent a reaction to the protein G of *Streptococci*. However, at least, antigens which were reacted with this monoclonal antibody may be target antigens.

Suzuki et al [15] reported that *Haemophilus parainfluenzae* outer membrane antigens were deposited on the glomeruli of patients with IgAN. Considering that *S. aureus* and *H. parainfluenzae* are both common oral and nasal flora, we speculate that both of these bacteria contribute to the pathogenesis of IgAN. In patients with IgAN, macroscopic hematuria frequently occurs suddenly after upper respiratory infection. It may indicate that bacterial antigens invasion occurs via injured mucosal barrier soon after upper respiratory infection, and the fragment of bacterial antigens and their antibodies presented in the sera may form circulating immune complexes, and formed immune complexes may deposit in the glomeruli.

In order to prevent the occurrence and progression of IgAN, new types of antibiotics that can eradicate such bacterial colonies and that can be taken over the long-term are needed. Presently, there are no suitable antibiotics for long-term prevention of *S. aureus* colonization [33, 34]. Another strategy would be to develop a vaccine that prevents colony formation without production of nephritogenic immune complexes [35].

For all of the reasons discussed above, it is important to clarify the mucosal and systemic immune status and genetic background of patients with IgAN. We demonstrated here that in a significant number of patients with IgAN, the antigen deposited on glomeruli was an *S. aureus* cell envelope protein, 'probable adhesin,' which may originate from colonized bacteria, and that it plays a key role in the immunopathogenesis of IgAN.

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REFERENCES

1. Lamm ME: Interaction of antigens and antibodies at mucosal surfaces. *Annu Rev Microbiol* 51:311–340, 1997

2. Krikler SJ, Lambert PA: Extracellular protein as a potential marker of active *Staphylococcus aureus* infection in bone. *J Med Microbiol* 37:227–231, 1992
3. Kluytmans J, van Belkum A, Verbrugh H: Nasal carriage of *Staphylococcus aureus*: Epidemiology, underlying mechanism, and associated risks. *Clin Microbiol Rev* 10:505–520, 1997
4. Marrack P, Kappler J: The *staphylococcal* enterotoxins and their relatives. *Science* 246:705–711, 1990
5. Herman A, Kappler JW, Marrack P, Pullen AM: Superantigens: Mechanism of T-cell stimulation and role in immune responses. *Annu Rev Immunol* 9:745–772, 1991
6. Silverman GJ, Goodyear CS: A model B-cell superantigen and the immunobiology of B lymphocytes. *Clin Immunol* 102:117–134, 2002
7. Davison AM: Infection-associated glomerulonephritis, in *Oxford Textbook of Clinical Nephrology*, edited by Davison AM, Cameron JS, Grunfeld JP, Kerr DNS, Ritz E, Winearls CG, Oxford, Oxford University Press, 1998, pp 667–687
8. Koyama A, Kobayashi M, Yamaguchi N, *et al*: Glomerulonephritis associated with MRSA infection: A possible role of bacterial superantigen. *Kidney Int* 47:207–216, 1995
9. Yoh K, Kobayashi M, Yamaguchi N, *et al*: Cytokines and T-cell responses in superantigen-related glomerulonephritis following methicillin-resistant *Staphylococcus aureus*. *Nephrol Dial Transplant* 15:1170–1174, 2000
10. Muro K, Yamagata K, Kobayashi M, *et al*: The usage of T cell receptor variable segments of the β chain in IgA nephropathy. *Nephron* 92:56–63, 2002
11. D'Amico G: The commonest glomerulonephritis in the world: IgA nephropathy. *Q J Med* 64:709–727, 1987
12. Koyama A, Igarashi M, Kobayashi M, *et al*: Natural history and risk factors for immunoglobulin A nephropathy in Japan. *Am J Kidney Dis* 29:526–532, 1997
13. Schena FP: Immunopathogenic aspects of primary IgA nephropathy. *Kidney Int* 48:1198–2013, 1995
14. Iwama H, Horikoshi S, Shirato I, Tomino Y: Epstein-Barr virus detection in kidney biopsy specimens correlates with glomerular mesangial injury. *Am J Kidney Dis* 32:785–93, 1998
15. Suzuki S, Nakatomi Y, Sato H, *et al*: *Haemophilus parainfluenzae* antigen and antibody in renal biopsy samples and serum of patients with IgA nephropathy. *Lancet* 345:12–15, 1994
16. Sato M, Kojima H, Takayama K, Koshikawa S: Glomerular deposition of food antigens in IgA nephropathy. *Clin Exp Immunol* 73:295–299, 1998
17. Yokoyama WM: Production of monoclonal antibodies, in *Current protocols in immunology*, edited by Coliga JE, Kruisebeel AM, Marugulies DH, Shevach EM, Strober W, National Institute of Health, New York, Wiley-Interscience, 1992, pp 2.5.1–2.5.17
18. Altschul SF, Stephen F, Thomas L, *et al*: Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402, 1997
19. Kuroda M, Ohta T, Uchiyama I, *et al*: Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 357:1225–1240, 2001
20. Foster TJ, Hook M: Surface protein adhesions of *Staphylococcus aureus*. *Trends Microbiol* 6:484–488, 1998
21. Cederholm B, Wieslander J, Bygren P, Heinegard D: Circulating complexes containing IgA and fibronectin in patients with primary IgA nephropathy. *Proc Natl Acad Sci USA* 85:4865–4868, 1988
22. Baldree LA, Wyatt RJ, Julian BA, *et al*: Immunoglobulin A-fibronectin aggregate levels in children and adults with Immunoglobulin A nephropathy. *Am J Kidney Dis* 22:1–4, 1993
23. Hiki Y, Iwase H, Kokubo T, *et al*: Association of asialo-galactosyl β1-3N-acetylgalactosamine on the hinge with a conformational instability of Jacalin-reactive immunoglobulin A1 in immunoglobulin A nephropathy. *J Am Soc Nephrol* 7:955–960, 1996
24. Allen AC, Bailey EM, Barratt J, *et al*: Analysis of IgA1 O-glycans in IgA nephropathy by florophore-assisted carbohydrate electrophoresis. *J Am Soc Nephrol* 10:1763–1771, 1999
25. Kokubo T, Hiki Y, Iwase H, *et al*: Evidence for involvement of IgA1 hinge glycopeptide in the IgA1-IgA1 interaction in IgA nephropathy. *J Am Soc Nephrol* 8:915–919, 1997
26. Kokubo T, Hiki Y, Iwase H, *et al*: Protective role of IgA1 glycans against IgA1 self-aggregation and adhesion to extracelluar matrix proteins. *J Am Soc Nephrol* 9:2048–2054, 1998
27. Tomana M, Matousovic K, Julian BA, *et al*: Galactose-deficit IgA1 in sera of IgA nephropathy patients is present in complexes with IgG. *Kidney Int* 52:509–516, 1997
28. Tomana M, Novak J, Julian BA, *et al*: Circulating immune complexes in IgA nephropathy consist of IgA1 with galactose deficit hinge region and antiglycan antibodies. *J Clin Invest* 104:73–81, 1999
29. Hiki Y, Kokubo T, Iwase H, *et al*: Underglycosylation of IgA1 hinge plays a certain role for its glomerular deposition in IgA nephropathy. *J Am Soc Nephrol* 10:760–769, 1999
30. Gharavi AG, Yan Y, Scolari F, *et al*: IgA nephropathy, the most common cause of glomerulonephritis, is linked to 6q22–23. *Nat Genet* 26:354–357, 2000
31. Holdsworth ST, Kitching R, Tipping P: Th1 and Th2 T helper cell subsets affect patterns of injury and outcomes in glomerulonephritis. *Kidney Int* 55:1198–1216, 1999
32. Ebihara I, Hirayama K, Yamamoto S, *et al*: Th2 predominance at the single-cell level in patients with IgA nephropathy. *Nephrol Dial Transplant* 16:1783–1789, 2001
33. Hudson IRB: The efficacy of intranasal mupirocin in the prevention of *Staphylococcal* infections: A review of recent experience. *J Hosp Infect* 27:81–98, 1994
34. Doebbeling BN, Reagan DR, Pfaller MA, *et al*: Long-term efficacy of intranasal mupirocin ointment. *Arch Intern Med* 154:1505–1508, 1994
35. Mckenney D, Pouliot KL, Wang Y, *et al*: Broadly protective vaccine for *Staphylococcus* based on an in vivo-expressed antigen. *Science* 284:1523–1527, 1999